

Inhibition of Rabies Virus Transcription in Rat Cortical Neurons with the Dissociative Anesthetic Ketamine

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In a previous study (B. P. Lockhart, H. Tsiang, P. E. Ceccaldi, and S. Guillemer, *Antiviral Chem. Chemother.* 2:9–15, 1991), we demonstrated an antiviral effect of the general anesthetic ketamine for rabies virus in neuronal cultures and in rat brain. This report describes an attempt to determine at what level ketamine acts on the rabies virus cycle in rat cortical neuron cultures. Immunofluorescence and [³⁵S]methionine labelling of infected neurons showed that ketamine (1 to 1.5 mM) inhibited viral nucleoprotein and glycoprotein syntheses. Northern (RNA) blots of total RNA from drug-treated neurons, hybridized with ³²P-labelled oligonucleotide probes for rabies virus nucleoprotein, matrix protein, and glycoprotein genes, showed a marked reduction (5- to 11-fold) in the levels of rabies virus mRNAs, relative to those in untreated neurons. No significant change in the levels of cellular β -actin mRNA were detected in ketamine-treated cells. A similar antiviral effect was observed with MK-801; however, no inhibition of rabies virus synthesis was observed with the general anesthetic chloral hydrate. The antiviral effect was not complete; a time-dependent recovery of viral transcription and rabies virus protein synthesis was observed, but no infectious virus was released into the culture supernatant. The lack of any modification of cellular protein or mRNA synthesis by ketamine suggests an antiviral mechanism acting at the level of rabies virus genome transcription.

Rabies virus, a member of the *Rhabdoviridae* family, contains an unsegmented negative-stranded RNA genome that codes sequentially for five proteins: nucleoprotein N, phosphoprotein M₁, matrix protein M₂, glycoprotein G, and RNA-dependent RNA polymerase L (29). The virus is highly neurotropic (38) and produces dramatic clinical symptoms leading invariably to death (12). Despite widespread and effective vaccination programs, rabies is still believed responsible for up to 20,000 human fatalities per year (35). The classical postexposure treatment for rabies infection consists of vaccination and, in cases of severe and advanced exposure, vaccination is combined with immunotherapy with either equine or human antirabies immunoglobulins (37). However, the limited availability of serum, combined with the potential risk of hepatitis B and human immunodeficiency virus contamination from human sera, has restricted its application and partly contributed to failures of postexposure treatment for rabies (2, 6, 36). It is for these reasons as well as for the lack of efficient anti-rabies virus agents (8, 11) that we were prompted to assess alternative potential drugs for effective postexposure treatment for rabies.

We previously showed that the dissociative anesthetic ketamine, a noncompetitive antagonist of the *N*-methyl-D-aspartate (NMDA) receptor, inhibited the production (100- to 1,000-fold) of rabies virus in a dose-dependent manner and that the inhibitory effect was unrelated to any mechanism operating through high-affinity NMDA receptor sites (20). The antiviral effect was highly selective for the class of noncompetitive NMDA receptor antagonists binding to the phencyclidine site, because other drugs of this class (MK-801 and phencyclidine derivatives) produced similar antiviral effects (32, 34). In this study, we attempted to determine which step of the rabies virus replicative cycle in neuronal

target cells is inhibited by ketamine so that future possible anti-rabies virus strategies may be developed.

MATERIALS AND METHODS

Infection of cells. Cortical neurons prepared from 16-day-old rat embryos as previously described (20) were seeded into 35- or 60-mm poly-L-ornithine-coated petri dishes and maintained in F-10 medium (GIBCO) supplemented with 7.5% fetal calf serum (Boehringer) and 7.5% horse serum (Boehringer). Five-day-old cultures were infected with 10⁶ PFU of fixed, BHK cell-adapted standard challenge rabies virus per ml, prepared as described by Tsiang et al. (33), for 1 h at 37°C. Unabsorbed virus was removed by washing, and fresh medium with or without drugs was added immediately. Extracellular rabies virus titers were assayed by plaque titration (26). Ketamine [1,2-(*o*-chlorophenyl)-2-methylene-aminocyclohexan-one] and MK-801 (5-methyl-10,11-dihydro-5H-dibenzo-(a,d)-cyclohepten-5,10-imine) were obtained from Research Biochemicals Inc.

Fluorescence microscopy. Cell cultures were washed with phosphate-buffered saline (PBS [pH 7.4]) and fixed with 80% acetone at –20°C for 20 min. The cells were then incubated with a mouse anti-rabies virus glycoprotein monoclonal antibody (1:50) in PBS for 1.5 h, washed, and treated with trimethylrhodamine isothiocyanate (TRITC)-labelled goat anti-mouse immunoglobulin G (1:20; Sigma) at 37°C for 1.5 h. Fluorescein isothiocyanate (FITC)-labelled rabbit anti-rabies virus nucleocapsid immunoglobulin G (1:20; Pasteur Diagnostics) was applied for 45 min at 37°C, and the cells were washed in PBS and covered with 1 drop of mounting medium (Citifluor). Samples were viewed with an inverted Zeiss microscope and photographed (Kodak TMY 400-ASA film) under UV illumination with the standard FITC or TRITC filter set. For immunofluorescence labelling of cell surface viral glycoprotein, unfixed cells were used and the procedure for glycoprotein staining was repeated with pre-

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warmed PBS containing Ca^{2+} (1.46 mM) and Mg^{2+} (2.16 mM).

[^{35}S]methionine labelling and immunoprecipitation of viral proteins. Infected cortical neurons were labelled with L-[^{35}S]methionine (10 $\mu\text{Ci ml}^{-1}$; Amersham) in methionine-deficient Dulbecco's minimal essential medium containing 7.5% fetal calf serum and 7.5% horse serum. Culture supernatants were removed at 24-h intervals and centrifuged at $500 \times g$ for 20 min. The supernatants were then centrifuged at $100,000 \times g$ for 1.5 h, and the pellets were solubilized in electrophoresis sample buffer (17). Cell extracts were immunoprecipitated with a rabbit anti-rabies virus nucleoprotein antibody as previously described (18). Samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (17), and gels were stained with Coomassie blue, dried, and exposed to Kodak X-Omat autoradiographic film for 7 to 14 days.

Preparation of oligonucleotide probes. DNA probes for rabies virus negative-stranded RNA were obtained by restriction enzyme cleavage from M13 plasmids containing rabies virus gene inserts as previously described (30). The N probe contained a *PvuII* insert (nucleotides 909 to 1041), the M_2 probe contained a *BamHI* insert (nucleotides 2709 to 2881), and the G probe contained an *AccI* insert (nucleotides 4584 to 4774); numbering is as for the PV strain of rabies virus (30, 31). The actin probe was prepared from plasmid pBR322 containing a 1.2-kb *PstI* insert coding for mouse β -actin as previously described (10).

Northern (RNA) blotting and hybridization of viral RNAs. Extraction of viral RNA from standard challenge rabies virus-infected cortical neurons seeded in petri dishes (60 mm) was based on the method of Sacramento et al. (24). Nucleic acid extracts were treated with DNase I (0.05 U μl^{-1} ; Promega) for 20 min at 37°C , and the RNA was recovered by extraction with phenol-chloroform (50:50) and then with chloroform and precipitated at -20°C in 0.3 M sodium acetate (pH 5.2) in 2.5 volumes of ethanol. Samples were denatured in 10 mM $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 7.4) containing 0.5 mM EDTA and 50% formamide for 5 min at 65°C and then separated by electrophoresis on a 1% (wt/vol) agarose gel containing 10% (vol/vol) formaldehyde. The RNA was transferred onto Hybond-N membranes (Amersham), fixed by exposure to UV irradiation for 5 min, and then heated at 85°C for 2 h. The membranes were prehybridized for 20 min at 65°C in 0.5 M $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 7.4) containing 1 mM EDTA and 7% SDS, hybridized with selective probes for 20 h at 65°C , washed in 40 mM $\text{NaH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$ (pH 7.4) containing 1 mM EDTA and 1% SDS (once at 55°C and twice at 65°C for 10 min each time), and exposed to Kodak X-Omat autoradiographic film for 3 to 9 days. Quantitative results of autoradiography were obtained by computer image analysis (PDI Quantity I software).

RESULTS

Cultures of rat cortical neurons were distinguishable as refringent cells extending neurites at the surface of a monolayer of nonneuronal cells consisting mainly of glial cells. Analysis of infected culture supernatants revealed that extracellular titers at 48 h postinfection (p.i.) were 6.9×10^5 PFU ml^{-1} in untreated cells and 5.7×10^2 PFU ml^{-1} in 1.5 mM ketamine-treated cells. Immunofluorescence labelling of viral nucleocapsids, after treatment of infected cortical neurons with an identical concentration of ketamine, indicated a marked reduction in the number of intracellular viral inclu-

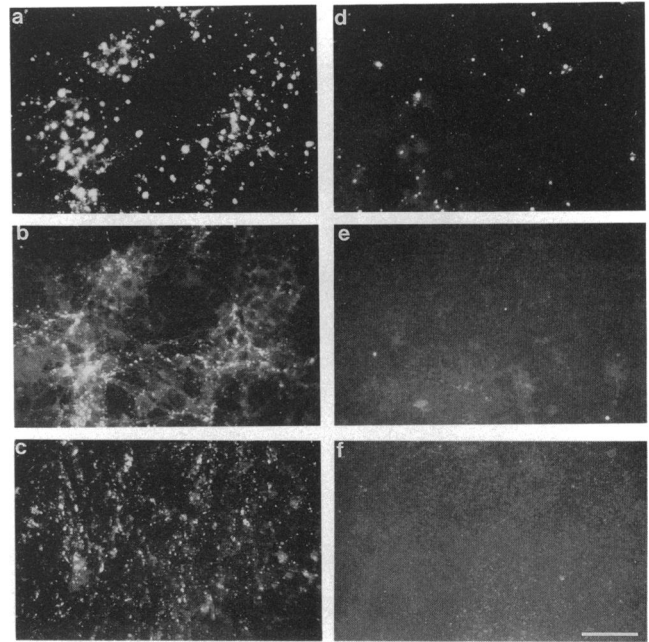


FIG. 1. Immunofluorescence localization of rabies virus nucleocapsids and glycoproteins in untreated (a to c) and 1.5 mM ketamine-treated (d to f) cortical neurons at 48 h p.i. Cells were fixed and labelled with FITC-anti-rabies virus nucleocapsid (a and d) and then successively with a mouse FITC-anti-rabies virus glycoprotein monoclonal antibody and TRITC-goat anti-mouse immunoglobulin G (b and e); unfixed cells were stained for surface viral glycoproteins (c and f) by immunofluorescence labelling with anti-rabies virus glycoprotein. Bar, 100 μm .

sions compared with that in control infected cells (Fig. 1a and d). Similarly, intracellular and cell surface viral glycoproteins were virtually undetectable in ketamine-treated virus-infected cells (Fig. 1e and f) compared with untreated virus-infected neurons (Fig. 1b and c).

To determine whether the antiviral effect was related to a modification in viral transcription, we determined the levels of rabies virus mRNA transcripts produced in the presence of 1.5 mM ketamine relative to those produced in untreated infected controls. Quantitative analysis of autoradiograms from Northern blots demonstrated that in ketamine-treated cells, the levels of viral N, M_2 , and G gene transcripts were 5-, 11-, and 6-fold lower, respectively, than equivalent control levels at 48 h p.i. (Fig. 2a). In contrast, ketamine had no effect on overall cellular transcription, as no decrease in the level of cellular β -actin mRNA was observed (Fig. 2a). Similarly, MK-801 reduced rabies virus N mRNA transcription (over 4-fold; Fig. 2b) as well as the levels of viral nucleocapsids and extracellular rabies virus titers (approximately 1,000-fold; data not shown). On the other hand, the general anesthetic chloral hydrate, when used at an identical concentration, caused no significant reduction of any of the above (Fig. 2b and data not shown).

To quantify as a function of time the inhibition of rabies virus production, we compared the levels of viral protein synthesis in cells treated with ketamine with those in untreated cells during a 72-h incubation period. A progressive increase in the number of viral nucleoprotein inclusions, detected by immunofluorescence microscopy, was observed in control infected cells from 24 to 72 h p.i. (Fig. 3). The antiviral activity of ketamine was demonstrated by the fact

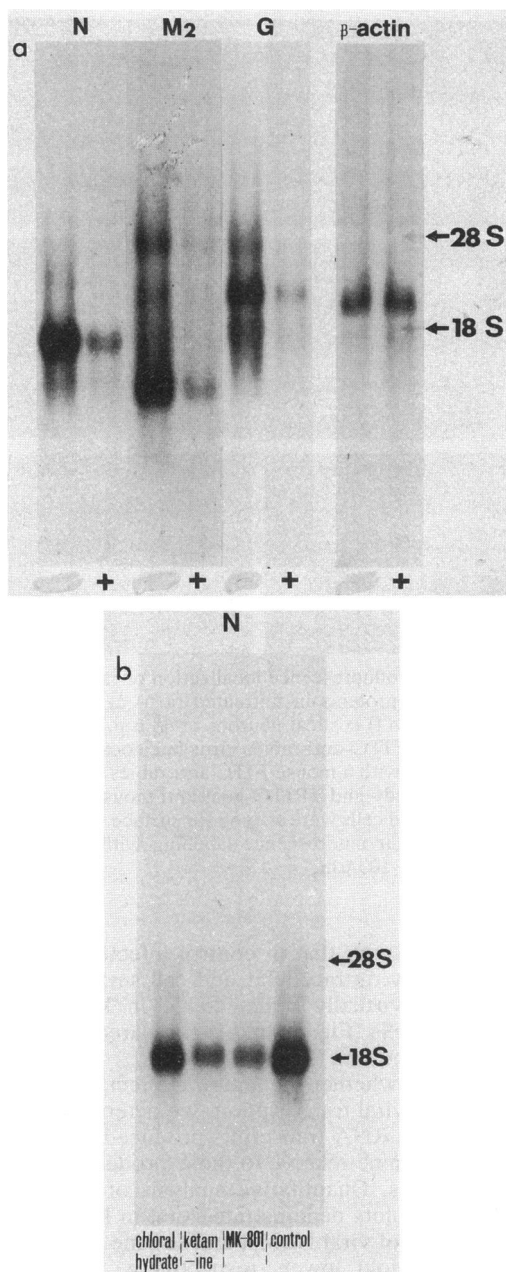


FIG. 2. Effects of ketamine and MK-801 on viral and cellular gene transcription. (a) Northern blot of total RNA ($1 \mu\text{g sample}^{-1}$) from infected untreated and 1.5 mM ketamine-treated (+) cortical neurons (48 h p.i.) hybridized with ^{32}P -labelled oligonucleotide probes for rabies virus: nucleoprotein N ($4.06 \times 10^5 \text{ cpm ml}^{-1}$), matrix protein M₂ ($3.25 \times 10^5 \text{ cpm ml}^{-1}$), glycoprotein G ($9.7 \times 10^4 \text{ cpm ml}^{-1}$), and cellular β -actin ($2.8 \times 10^5 \text{ cpm ml}^{-1}$). (b) Total RNA ($2.5 \mu\text{g sample}^{-1}$) from infected cortical neurons treated with a 1 mM concentration of the indicated drugs for 48 h was hybridized with a rabies virus N probe ($4.2 \times 10^5 \text{ cpm ml}^{-1}$). Mock-infected cells were uniformly negative for rabies virus probes. Autoradiograms were developed after 4 to 9 days.

that the numbers of viral inclusions at 24, 48, and 72 h p.i. represented only 22, 23, and 33% of control values, respectively.

Immunoprecipitation of ^{35}S -labelled viral proteins indi-

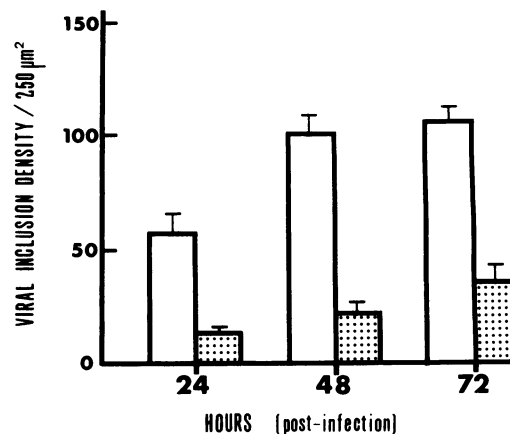


FIG. 3. Viral nucleocapsid inclusion density in cortical neurons. The number of fluorescent inclusions in the cytoplasm of cells was estimated at four different regions of the culture dish for three separate experiments. Symbols: □, infected control cells; ▨, 1 mM ketamine-treated cells. Values represent the mean scoring density plus the standard error of the mean. Background scores averaged two inclusions per $250 \mu\text{m}^2$.

cated a twofold increase in the levels of intracellular viral nucleoproteins from 24 to 72 h p.i. (Fig. 4). This increase correlated with a twofold increase in the amounts of N gene transcripts (Fig. 5). Ketamine, on the other hand, considerably reduced the levels of both viral nucleoproteins (two- to threefold) and N gene transcripts (three- to fivefold) relative to those in control infected cells (Fig. 4). However, in drug-treated cells, the levels of viral nucleoproteins and N gene transcripts also increased (threefold) from 24 to 72 h p.i., although the amounts at 72 h p.i. were still almost twofold lower than those in untreated virus-infected cells at

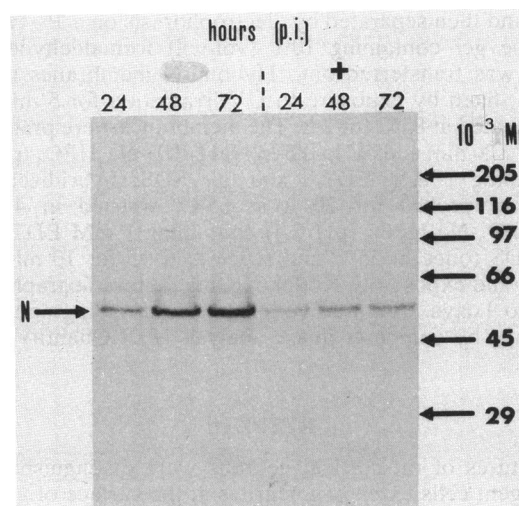


FIG. 4. Effect of ketamine on viral protein synthesis. At 24, 48, and 72 h p.i., infected cortical neurons labelled with ^{35}S methionine ($10 \mu\text{Ci ml}^{-1}$) in the absence or (+) presence of 1 mM ketamine were immunoprecipitated with an anti-rabies virus nucleoprotein antibody. Samples were separated by SDS-polyacrylamide gel electrophoresis and exposed to autoradiographic film for 5 days. An equal amount of cell extract ($50 \mu\text{g}$) was immunoprecipitated, and an identical volume was loaded onto each lane.

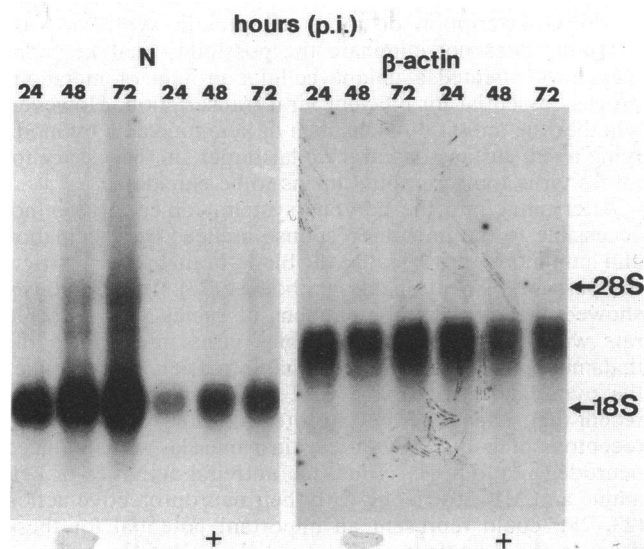


FIG. 5. Northern blot of total mRNA ($5 \mu\text{g sample}^{-1}$) from infected untreated or 1 mM ketamine-treated (+) cortical neurons hybridized at 24 to 72 h p.i. with rabies virus nucleoprotein N ($3.6 \times 10^5 \text{ cpm ml}^{-1}$) and cellular β -actin ($2.8 \times 10^5 \text{ cpm ml}^{-1}$) probes. Mock-infected controls were uniformly negative. Autoradiograms were developed after 5 to 9 days.

24 h p.i. (Fig. 5). No change in the levels of β -actin mRNA was observed at 24 h p.i. in infected untreated and drug-treated cells. A fivefold decrease in rabies N mRNA levels from 48 to 72 h p.i. in treated cells coincided with a threefold increase in the synthesis of rabies virus N mRNA (Fig. 5). Despite this increase in the levels of viral N mRNA and nucleoprotein in the presence of ketamine, rabies virus titers at 72 h p.i. ($5.4 \times 10^4 \text{ PFU ml}^{-1}$) were over 100-fold lower than control values ($7.8 \times 10^6 \text{ PFU ml}^{-1}$) and remained as such up to 96 h p.i.

Furthermore, at 72 h p.i., infected cell cultures labelled with [^{35}S]methionine released complete rabies virus, con-

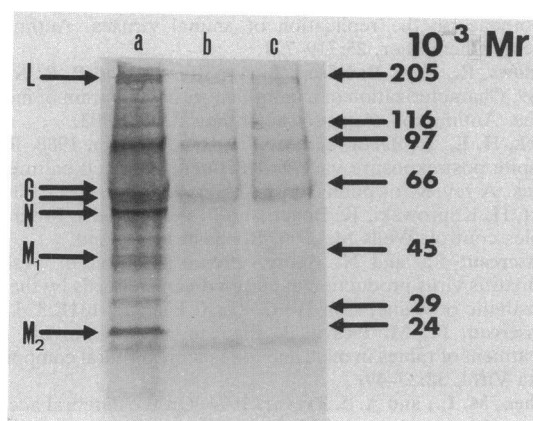


FIG. 6. Effect of ketamine on rabies virus protein synthesis. At 72 h p.i., supernatants (2.5 ml) from infected cortical neurons labelled with [^{35}S]methionine ($10 \mu\text{Ci ml}^{-1}$) were concentrated by centrifugation, analyzed by SDS-polyacrylamide gel electrophoresis, and exposed to autoradiographic film for 9 days. (a) Control. (b) Infected cells treated with 1 mM ketamine. (c) Mock-infected cells. Viral proteins were located by reference to the migration of purified standard challenge rabies virus.

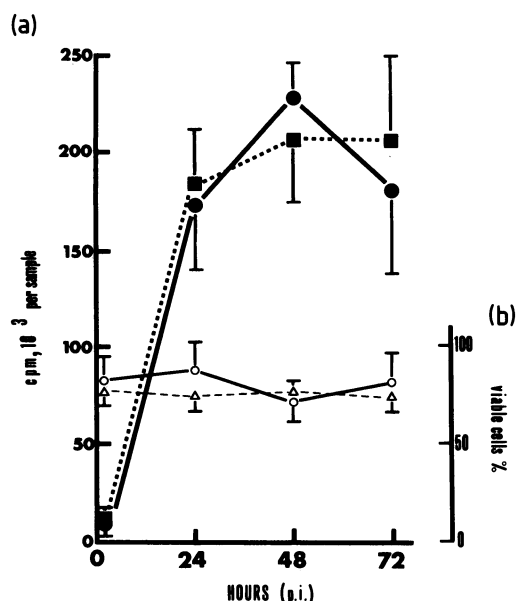


FIG. 7. (a) Effect of ketamine on [^{35}S]methionine incorporation into trichloroacetic acid-precipitable material. Infected untreated (●) or 1 mM ketamine-treated (■) cortical neurons were incubated in Dulbecco's minimal essential medium (minus methionine) containing [^{35}S]methionine ($2.5 \mu\text{Ci ml}^{-1}$). At selected times, cells were scraped from the culture dish (30 mm), washed twice in PBS, and then precipitated with an equal volume of 20% (wt/vol) trichloroacetic acid. Pellets were washed in acetone and solubilized in a universal scintillant (Packard). Data quoted represent the mean of triplicate samples \pm the standard error of the mean (SEM). (b) Infected neurons maintained in F-10 medium containing 7.5% fetal calf serum and 7.5% horse serum in the absence (○) or the presence (△) of 1 mM ketamine were estimated from the number of viable cells (14) at three different regions of the culture dish for two samples. Data represent the mean \pm the SEM.

taining all five viral proteins, into the culture supernatant (Fig. 6). However, with ketamine, no extracellular viral proteins were detected, as in mock-infected cells (Fig. 6).

The concentration of ketamine (1 to 1.5 mM) used in the present study did not inhibit total cellular protein synthesis, as analyzed by the incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material, because no significant difference was observed relative to controls (Fig. 7a). Similarly, cell morphology or viability (Fig. 7b), as assessed by phase-contrast microscopy or a sensitive histofluorescence staining procedure (15), respectively, was unaffected. These results are further evidence that the inhibition of rabies virus replication with ketamine was not related to any relative inhibition of cellular functions.

DISCUSSION

The present study demonstrated a novel antiviral effect of the general anesthetic ketamine against rabies virus in rat primary cortical neurons; the effect was not a virucidal or cytopathic one. Furthermore, under our conditions of inoculation, it is unlikely that ketamine had any effect on the early events of viral infection of neurons, such as binding, penetration, or uncoating. In addition, the antiviral effect was not related to an inhibition of cellular transcription or translation because (i) the expression of a "housekeeping" gene (β -actin) was unaffected by the drug treatment and (ii)

the inhibitory effects on viral mRNA and protein syntheses occurred to a similar extent, indicating that viral mRNA was fully translated. This result strongly suggests that the antiviral activity involves an inhibition of rabies virus genome transcription; however, an additional effect on viral genome replication cannot be ruled out. Present-day models of rhabdovirus transcription suggest a sequential decline in viral genome transcription from the 3'- to the 5'-encoded cistrons (3). On this basis, it is possible that the ketamine-mediated inhibition of viral mRNA transcription modifies rabies virus genome transcription by influencing the amounts of rabies virus mRNAs. However, the inhibition is not complete, and a time-dependent recovery in viral transcription and protein synthesis is observed. The mechanism by which this recovery occurs is not known, but it is possible that inactivation of ketamine by ionization and/or metabolism to an N-methylated product (9) parallels the recovery in rabies virus genome transcription. This suggestion is supported by the fact that we observed no increase in extracellular rabies virus titers or the synthesis of viral nucleocapsids when fresh medium containing ketamine (1.5 mM) was added daily for up to 7 days p.i. (34).

The antiviral mechanism of ketamine appears unrelated to any effect on cellular cytoskeletal organization, because we observed no gross morphological changes in neuronal structure and, furthermore, the disruption of neuronal actin filaments does not affect rabies virus production (19). Similarly, a ketamine-mediated modification in channel-mediated transmembrane ion flux is unlikely to inhibit viral synthesis because the Na⁺ and Ca²⁺ channel antagonists amiloride (1 to 25 μ M) (34) and nifedipine (1 to 10 μ M) (19), respectively, do not affect rabies virus production.

In a previous study, we demonstrated that MK-801 inhibited rabies virus infection in rat primary cortical neurons (32). Here we have shown that the antiviral effect involves an inhibition of rabies virus genome transcription similar to that observed with ketamine. However, it is uncertain whether the inhibitory effect is related to the anesthetic or other specific functional properties of these drugs. It was previously shown that high concentrations of local anesthetics possess antiviral activities, but in those cases, the inhibitory effect was likely to have resulted from a drug-induced virucidal effect (39) or an inhibition of cellular protein synthesis (7). It has been reported that volatile general anesthetics possess antiviral activities, probably by blocking viral RNA synthesis (15, 16). For example, halothane differentially inhibits the replication of a number of RNA- and DNA-containing viruses (4, 5). In contrast, in the present study, the general anesthetic chloral hydrate had no effect on rabies virus synthesis; however, the lack of an antiviral effect with chloral hydrate could be due to the fact that it is clinically less potent than ketamine. Furthermore, the antiviral effects of ketamine and MK-801 are virus selective, because the replication of herpes simplex virus, vesicular stomatitis virus, poliovirus type 1, and human immunodeficiency virus type 1 was not inhibited by either anesthetic (32, 34).

It is well established that general anesthetics can directly interact with membrane proteins and thereby inhibit their function (27). Furthermore, because noncompetitive NMDA receptor antagonists have no difficulty penetrating the cytoplasm of neurons (13), ketamine may act on an intracellular target. Specific intracellular proteins, notably, β -tubulin (21), or metabolic systems, such as ATP levels (28), were previously shown to regulate viral transcription. Consequently, the lack of any significant modification in cellular

β -actin transcription or total cell protein synthesis with ketamine does not eliminate the possibility that ketamine may have affected a unique cellular protein or metabolic process essential for efficient viral transcription. However, whether the antiviral mechanism of ketamine acts by modifying a cell surface or intracellular target site necessary for rabies virus transcription remains to be elucidated.

After entry into the nervous system, rabies virus is not accessible to the immune response induced by vaccination and immunotherapy because the blood-brain barrier restricts the passage of neutralizing antibodies (22). We previously showed that peripheral treatment of rabies virus-infected rats with ketamine reduced rabies virus infection in the thalamus, cortex, and hippocampal formation (in particular, the pyramidal layer of the CA1 region) (20). Similarly, a recent report has described a protective effect of the NMDA receptor antagonist MK-801 against measles virus-induced neurodegeneration (1). Thus, the antiviral activities of ketamine and MK-801, along with their neuroprotective action (23, 25), could represent an important potential for these drugs in the treatment of advanced clinical rabies.

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